AD-A266 511

REPORT DOCUMENTATION PAGE

1b. RESTRICTIVE MARKINGS	
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution is unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S): NMRI 93-41	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Naval Medical Research Institute	6b. OFFICE SYMBOL (If applicable)
7a. NAME OF MONITORING ORGANIZATION: Naval Medical Command	
6c. ADDRESS (City, State, and ZIP Code): 8901 Wisconsin Avenue, Bethesda, MD 20889-5055	7b. ADDRESS (City, State, and ZIP Code): Department of the Navy, Washington, DC 20372-5120
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: Naval Medical Research & Development Command	8b. OFFICE SYMBOL (If applicable)
9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8c. ADDRESS (City, State, and ZIP Code): 8901 Wisconsin Avenue, Bethesda, MD 20889-5044	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO	PROJECT NO	TASK NO	WORK UNIT ACCESSION NO.
62787A	3M162787	A870.AR1288	DN243536

11. TITLE (Include Security Classification)
Detection of West Nile virus by the polymerase chain reaction and analysis of nucleotide sequence variation

12. PERSONAL AUTHOR(S) Porter KR; Summers PL, Dubois D, Puri B, Nelson W, Henchal E, Oprandy JJ, Hayes CG

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
journal article	FROM _____ TO _____	1993	7

16. SUPPLEMENTARY NOTATION
Reprinted from: American Journal of Tropical Medicine and Hygiene 1993 Vol.48 No.3 pp. 440-446

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	diagnostic tests; viral diseases; flaviviruses; polymerase chain reaction; Japanese encephalitis

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Phyllis Blum, Librarian	22b. TELEPHONE (Include Area Code): (301) 295-2188 / 22c. OFFICE SYMBOL: MRL/NMRI

Am. J. Trop. Med. Hyg., 48(4), 1993, pp. 445–448

DETECTION OF WEST NILE VIRUS BY THE POLYMERASE CHAIN REACTION AND ANALYSIS OF NUCLEOTIDE SEQUENCE VARIATION

KEVIN R. PORTER, PETER L. SUMMERS, DORIA DUBOIS, BEENA PURI, WILLIAM NELSON, ERIK HENCHAL, JOHN J. OPRANDY, AND CURTIS G. HAYES

Infectious Diseases Threat Assessment Program, Naval Medical Research Institute, Bethesda, Maryland; Walter Reed Army Institute of Research, Washington, District of Columbia

Abstract. A polymerase chain reaction (PCR) assay was developed to rapidly detect and identify West Nile (WN) virus. The RNA from seven isolates of WN virus from six countries and four other flaviviruses (Kunjin, Japanese encephalitis, St. Louis encephalitis, and yellow fever viruses) was reverse-transcribed (RT) and amplified by PCR. The nucleotide sequences of the amplified products were determined by a rapid, automated DNA sequencing method. The WN virus RT/PCR assay detected the target gene segment of isolates from both the African-Middle Eastern group and the Indian group with a sensitivity of approximately 0.05 pg of viral RNA. Kunjin virus was the only other flavivirus tested that produced a band of the appropriate size. Five of seven WN virus isolates showed 92–98% homology in the nucleotide sequence of their PCR products. The sequence of one isolate was virtually identical to the published sequence of the Nigerian isolate (99.5% homology). No correlation was established between the degree of nucleotide homology, geographic location, time of isolation, or source of the isolates.

West Nile (WN) virus belongs to the Japanese encephalitis complex of the Flaviviridae and is known to cause WN fever. Epidemics of WN fever in Israel, Egypt, France, and South Africa have resulted in thousands of human cases. The predominant vectors for the virus are *Culex* mosquitoes. Wild birds are the most common vertebrate hosts involved in the natural transmission cycle of WN virus.[1]

Methods available for the detection and identification of WN virus include mouse and cell culture inoculation, followed by serologic identification of isolates. However, the performance of these assays is laborious and requires days to weeks to complete. Thus, this study was conducted to explore the use of a more rapid procedure, the polymerase chain reaction (PCR) assay, to detect and identify WN virus.

MATERIALS AND METHODS

Virus stocks

Six isolates of WN virus, originally isolated from five different countries (Table 1), were kindly provided by Dr. Robert Shope (Yale University, New Haven, CT). A seventh isolate, B956, was obtained from the American Type Culture Collection (Rockville, MD). Isolates of Japanese encephalitis (JE) virus, St. Louis encephalitis (SLE) virus, Kunjin (KUN) virus, and yellow fever (YF) virus were provided by Dr. Charles Hoke (Walter Reed Army Institute of Research). The virus specimens were cultured in a C6/36 mosquito cell line. The growth medium for the cells was Eagle's minimal essential medium supplemented with 5% fetal bovine serum, 200 units/ml of penicillin, and 200 μg/ml of streptomycin. Confluent C6/36 cultures were inoculated with approximately 10^3 plaque-forming units (PFU) of each stock virus. After showing 80% cytopathic effects, the culture supernatants were harvested and stored at −80°C until used. Suckling mouse brain (SMB) preparations of each virus strain were made as described previously[2] and stored at −80°C. Viral infectivity titers for all culture supernatants and SMB preparations were determined by plaque assay in LLC-MK$_2$ cells and the identity of viral specimens was confirmed by plaque-reduction neutralization tests using viral-specific hyperimmune mouse ascitic fluid.[3]

Preparation of RNA

RNA was isolated from viral culture supernatants and suckling mouse brain preparations as previously described, with some modifica-

TABLE 1
Characteristics of West Nile virus isolates

Virus*	Passage	Source	Location	Date
68856	Vero cells	Bat	Sagar, India	1968
E101	Vero cells	Human serum	Egypt	1983
AN4766	SMB	Avian	Ethiopia	1970
AN4767	SMB	Avian	Ethiopia	1970
Dak B310	SMB	*Culex* sp.	Central African Republic	1967
Dak MG798	?	*Culex vasa*	Madagascar	1978
B956	C6/36 cells	Human serum	Uganda	1937

* 68856 = Indian group, E101, AN4766, AN4767, Dak B310, Dak MG798, and B956 = African-Middle Eastern group. SMB = sucking mouse brain preparation.

tions.[4] Briefly, 100 μl of culture supernatant or SMB preparation was sequentially mixed with 400 μl of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol), 50 μl of 2 M sodium acetate, 500 μl of water-saturated phenol, and 100 μl of 49:1 chloroform:isoamyl alcohol mixture. After a 15-min incubation on ice, the specimens were centrifuged at 10,000 × *g* for 5 min at 4°C. The aqueous layer was transferred to a new tube and ice-cold isopropanol was added to give a 1:1 volume. The samples were incubated for 1 hr at −20°C. The RNA was pelleted by centrifugation at 10,000 × *g* for 15 min at 4°C. The pellet was washed with 75% ethanol, vacuum-dried, and resuspended in diethylpyrocarbonate (DEPC)–treated water containing 10 units of human placental ribonuclease inhibitor (Sigma, St. Louis, MO). The isolated RNA was stored at −80°C until used. Aliquots of each viral specimen were plaque-assayed prior to RNA extraction to determine the titer of infectious virus in each sample.

Reverse transcription and polymerase chain reaction (RT/PCR)

The NS3 nonstructural gene of WN virus was chosen as the target for the PCR. A nineteen-base oligonucleotide primer, KP7 (5′ GCAGAGTGATCGACAGCCG 3′) and a 20-base primer, KP8I (5′ CCACCAGACCATTCGGCATG 3′), were designed to amplify a 258-base-pair (bp) fragment of the target gene.

In initial experiments, approximately 1 μg of RNA extracted from SMB preparations was used for the RT/PCR of WN virus B956, JE, SLE, and KUN. For the RT/PCR of YF virus, 1 μg of RNA isolated from culture supernatants was used. To evaluate sensitivity for different WN virus isolates, 10-fold dilutions of culture supernatant–derived viral RNA were made, ranging from 5 pg/μl to 0.05 pg/μl. One microliter of each dilution was used in the RT/PCR. The viral RNA was added to a 0.5-ml microfuge tube containing DEPC-treated water and 50 pmoles of primer KP8I and incubated at 68°C for 3 min. To this mixture was added 2 μl of 10× RT/PCR buffer (700 mM Tris pH 8.8, 200 mM $(NH_4)_2SO_4$, 10 mM dithiothreitol, 25 mM $MgCl_2$, 1% Triton X 100), 50 units of Superscript reverse transcriptase (Bethesda Research Laboratories, Grand Island, NY), 0.5 mM deoxynucleotide triphosphates, and 10 units of human placental ribonuclease inhibitor (Sigma). The total volume of the RT reaction was 20 μl. After incubation for 30 min at 37°C, 80 μl of PCR mixture (70.75 μl of distilled water, 8 μl of 10× RT/PCR buffer, 1 μl [50 pmoles] of antisense primer KP7, and 0.25 μl [1.25 units] of AmpliTaq DNA polymerase [Perkin-Elmer-Cetus, Norwalk, CT]) was added to each tube. Thirty-five PCR cycles were performed with each cycle consisting of denaturation at 95°C for 1 min, primer annealing at 58°C for 1 min, and primer extension at 70°C for 1 min. The PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining.

Sequencing of the PCR product

Primers KP7 and KP8I were labeled at the 5′-end with biotin molecules (KP7b and KP8Ib) and used to amplify RNA from each WN virus isolate. For each RNA specimen, two separate reactions were performed: one using primers KP7 and KP8Ib and the other using primers KP7b and KP8I. The double-stranded biotinylated products were attached to streptavidin-coated magnetic beads (Dynal Inc., Great Neck, NY).

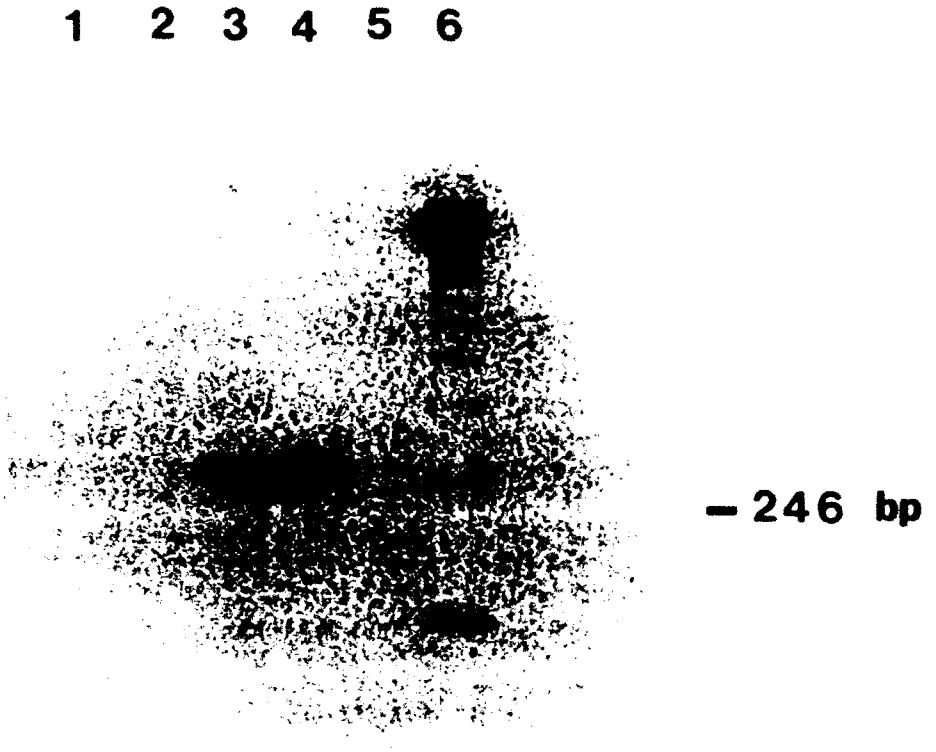

FIGURE 1. West Nile (WN) virus and control virus reverse transcription-polymerase chain reaction (RT-PCR) products electrophoresed on a 3% agarose gel stained with ethidium bromide. Lane 1, St. Louis encephalitis virus; lane 2, yellow fever virus; lane 3, Kunjin virus; lane 4, WN virus isolate B956; lane 5, C6/36 cell RNA; lane 6, molecular weight marker. Kunjin virus and WN virus show positive bands. The faint, low molecular weight band in lane 1 resulted from nonspecific priming. Japanese encephalitis virus failed to produce any bands. bp = basepairs.

The strands were denatured with NaOH and the unbiotinylated strands were removed, leaving sense- or antisense-strands attached to the beads depending on which primer was biotinylated. The nucleotide sequences of the attached strands were determined using a system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides.[5] Primer KP8I was used to sequence the sense-strand and primer KP7 was used to sequence the antisense-strand.

RESULTS

Specificity of the PCR

RNA was isolated from the Ugandan B956 isolate of WN virus and the JE, SLE, KUN, and YF viruses and used in the RT/PCR with primers KP7 and KP8I. A strong band of the predicted 258-bp size was seen in the WN virus sample. Japanese encephalitis, SLE, and YF viruses all failed to show any bands of the appropriate size (Figure 1). However, KUN virus produced a distinct band of the predicted size that reflected its high degree of genomic homology with WN virus.[6] The sensitivity of the assay was determined to be 6.5 PFU/100 μl of spiked human serum, using a membrane-based RNA isolation method.[7] The identity of the Ugandan B956 PCR product was confirmed as WN virus by sequence analysis (Figure 2).

Detection of different WN virus isolates

Six additional isolates of WN virus from five different countries were grown in C6/36 cells and the isolated RNA was used in RT/PCR. Five of the isolates belonged to the African-Middle East-

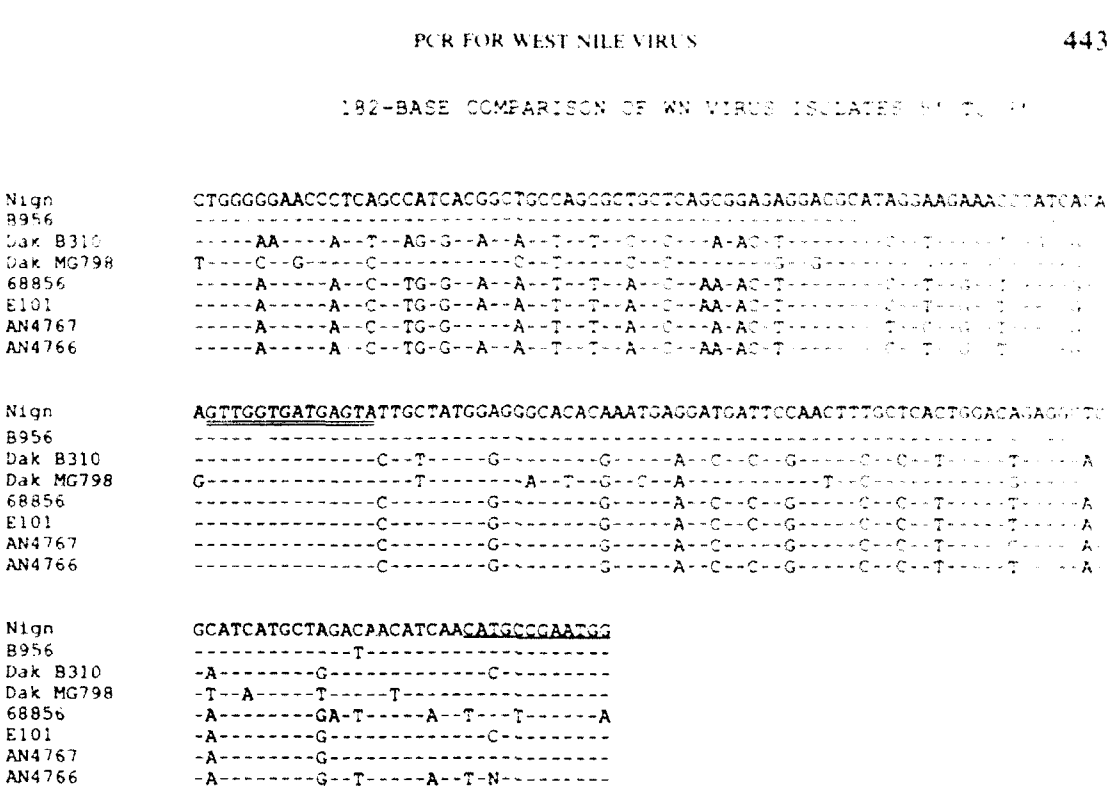

FIGURE 2. Comparison of 182 bases of the reverse transcriptase/polymerase chain reaction (RT/PCR) product nucleotide sequence from each isolate of West Nile (WN) virus. The top line represents the same 182-base region of the published Nigerian (Nign) sequence. *Nucleotide changes compared with the Nigerian sequence are* indicated by the appropriate letter. - indicates identical bases. The 5′ end of the KP8I primer-binding site is underlined and a conserved 14-base stretch is double underlined.

em (AME) group and one to the Indian group (Table 1). The assay was able to detect as little as 0.05 pg of viral RNA from all isolates. The Indian isolate 68856 did not amplify as efficiently as the AME isolates as indicated by less distinct bands (Figure 3).

Nucleotide sequence analysis

Biotinylated PCR products from each WN virus isolate were produced using KP7b and KP8Ib in combination with the unbiotinylated primers as described above. Of the 258-bp PCR product, 182 bases of nucleotide sequence were generated from each of the seven isolates for comparison (Figure 2). All seven WN virus isolates showed 68% nucleotide sequence homology. A stretch of 14 nucleotides was conserved among all isolates. Twelve bases of the 5′-end of the primer-binding site of isolate 68856 from India showed a 25% mismatch compared with a 0–16% mismatch for the AME isolates.

Sequence homology comparisons of each isolate are shown in Table 2. Isolates E101, 68856, AN4767, AN4766, and Dak B31O showed 92–98% homology in their PCR product nucleotide sequences. Each of these five isolates was only 77–82% homologous with the published Nigerian sequence.[8] Isolate B956 was virtually identical to the Nigerian sequence (99.5% homology). The Dak MG798 isolate showed only 73–86% homology with other WN virus isolates. No correlation was established between the degree of nucleotide sequence homology, geographic location, time of isolation, or source of the isolate.

The amino acid sequence for each 182-bp segment was determined and compared with the amino acid sequence of the published nucleotide sequence of the Nigerian isolate (Figure 4). Overall, there was 94–97% protein sequence homology. Substitutions occurred at positions 3, 7, 72, 76, and 77. The changes consisted of lysine for glutamic acid (Dak B31O), valine for isoleucine (68856, AN4766/67, Dak B31O, and E101), asparagine for aspartic acid (68856), threonine for methionine (E101 and Dak B31O), and serine for proline (68856).

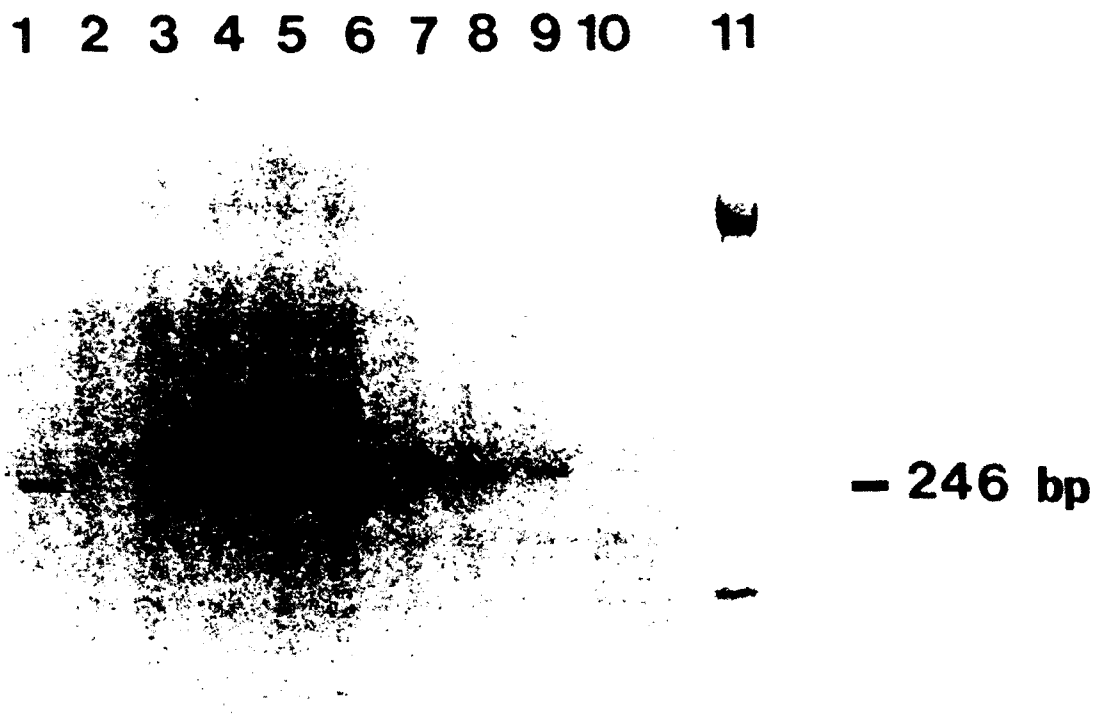

FIGURE 3. Reverse transcriptase/polymerase chain reaction (RT/PCR) products from different isolates of West Nile (WN) virus. Lanes 1–3, 68856; lanes 4–6, Dak MG798; lanes 7–9, Dak B310. Each series of three lanes represents the amplification of 5 pg, 0.5 pg, and 0.05 pg of viral RNA. Bands of the appropriate size were seen in all lanes containing WN virus PCR product. The WN virus isolates AN4767, AN4766, B956,and E101 showed similar results. Lane 10 contains the RT/PCR product from C6/36 cell RNA. Lane 11 contains the molecular weight marker. bp = basepairs.

DISCUSSION

A sensitive and specific PCR-based method was developed for the detection and identification of WN virus. No cross-reactions occur between WN virus PCR primers and JE, SLE or YF viruses. Cross-reactivity does occur with KUN virus. This is consistent with the results of Cocia and others, which showed that the percent homology between WN and KUN virus nucleotide and amino acid sequences was 79% and 93%, respectively.[6]

West Nile virus has been categorized into two distinct antigenic groups.[9,10] The AME group consists of isolates from Africa, Israel, Pakistan, France, and the former Soviet Union. The other

TABLE 2

*Individual nucleotide sequence comparisons for each West Nile virus isolate**

Isolate†	68856	AN4767	AN4766	B956	Dak B310	E101	Dak MG798
68856	100	93	98	77	92	96	76
AN4767	93	100	95	82	93	96	82
AN4766	98	95	100	79	93	98	77
B956	77	82	79	100	79	80	86
Dak B310	92	93	93	79	100	96	81
E101	96	96	98	80	96	100	79
Dak MG798	76	82	77	86	81	79	100
Nigerian	77	82	78	99.5	79	80	86

* Values indicate percent homology between the isolates compared.
† 68856 = Indian group; AN4767, AN4766, B956, Dak B310, E101, Dak MG798, and Nigerian = African-Middle Eastern group

COMPARISON OF TRANSLATED PROTEIN SEQUENCES FOR WN VIRUS ISOLATES

```
Nign        L G E P S A I T A A S A A Q R R G R I G R N P S Q V G D E Y C Y G G H T
68856       - - - - - - V - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
AN4766      - - - - - - V - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
AN4767      - - - - - - V - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Dak B310    - - K - - - V - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
B956        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
E101        - - - - - - V - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Dak MG798   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Nign        N E D D S N F A H W T E A R I M H T N E D D S N F A H W T E A R I M L D
68856       - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - N
AN4766      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
AN4767      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Dak B310    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
B956        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
E101        - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Dak MG798   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Nign        N I N M P -
68856       - - - - S -
AN4766      - - - X - -
AN4767      - - - - - -
Dak B310    - - - T - -
B956        - - - - - -
E101        - - - T - -
Dak MG798   - - - - - -
```

FIGURE 4. Comparison of the amino acid sequences translated from the 182 bases from each West Nile (WN) virus isolate. The top line shows the amino acid sequence deduced from the same 182-base region of the published Nigerian (Nign) sequence. Amino acid differences compared with the Nigerian sequence are indicated by the appropriate letter. - indicates identical amino acids.

group consists of viruses isolated from India. The primers used in the WN virus PCR assay were able to detect RNA from viruses in both antigenic groups despite mismatches at the primer-binding sites of each isolate. The Indian isolate had the most mismatches at the primer-binding site, which may explain the lower efficiency of amplification.

Variation, if any, in nucleotide sequences among WN virus isolates is not well defined. We sequenced the PCR product from each isolate to look for sequence differences among the seven isolates. Based on the degree of sequence homology, the isolates could be grouped into three categories. Group I isolates showed 92–98% homology and included E101, 68856, AN4767, AN4766, and Dak B31O. Group II isolates (B956 and Nigerian) were almost identical (99.5% homology). A single isolate, Dak MG798 from Madagascar, was placed in group III because it showed only 77–86% homology with the other isolates. In contrast to the antigenic differences between viruses in the AME group and the Indian group, the Indian isolate showed a high degree of nucleotide sequence homology with four of the six AME group viruses. In a mouse passive protection assay, the Indian and AME isolates were cross-protective (Dubois D, unpublished data), lending support to the similarity between the AME isolates and the Indian isolates analyzed in this study. Although these comparisons were made over a small portion of the WN virus NS3 gene, the sequence variations observed may be useful in the further categorization of WN virus isolates given the highly conserved nature of NS3 nonstructural proteins.

The translated amino acid sequences for the 182 bases of each WN virus isolate maintained a high degree of homology (94–97%). This indicates that most of the mutations seen among the viral isolates are silent mutations.

The RT/PCR assay described here is a sensitive, specific, and rapid method for the detection and characterization of WN virus. Field studies using the RT/PCR assay together with serology and virus isolation are needed to determine the clinical usefulness of this method. The RT/PCR and rapid sequencing of its products may be useful in epidemiologic studies of WN virus infections.

Financial support: This work was supported by the U.S. Naval Medical Research and Development Command work unit 3M162787A870AR122.

Disclaimer: The opinions and assertions contained herein are not to be construed as official or as reflecting the views of the Navy services at large.

Authors' addresses: Kevin R. Porter, Beena Puri, William Nelson, John J. Oprandy, and Curtis G. Hayes, Infectious Diseases Threat Assessment Program, Naval Medical Research Institute, Bethesda, MD 20852. Peter L. Summers, Doria Dubois, and Erik Henchal, Walter Reed Army Institute of Research, Washington, DC 20307-5100.

Reprint requests: Kevin R. Porter, Naval Medical Research Institute Annex, 12300 Washington Avenue, Rockville, MD 20852.

REFERENCES

1. Hayes CG, Baqar S, Ahmed T, Chowdhry MA, Reisen WK, 1982. West Nile virus in Pakistan. I. Seroepidemiological studies in Punjab Province. *Trans R Soc Trop Med Hyg 76:* 431–436.
2. Clarke DH, Casals J, 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Am J Trop Med Hyg 7:* 561–573.
3. Russell PK, Nisalak A, Sukhavachana P, Vivona S, 1967. A plaque reduction test for dengue virus neutralizing antibodies. *J Immunol 99:* 285–290.
4. Chomezynski P, Sacchi N, 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem 162:* 156–159.
5. Prober JM, Trainor BL, Dam RJ, Hobbs FW, Robertson CW, Zagursky RJ, Cocuzza AJ, Jensen, MA, Baumerster K, 1987. A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. *Science 238:* 336–341.
6. Cocia G, Parker MD, Spright G, Byrne ME, Westaway EG, 1988. Nucleotide and complete amino acid sequences of Kunjin virus: definitive gene order and characteristics of the virus-specified proteins. *J Gen Virol 69:* 1–21.
7. Porter KR, Polo SL, Long GW, Merritt SC, Oprandy JJ, 1991. A rapid membrane-based viral RNA isolation method for the polymerase chain reaction. *Nucleic Acids Res 19:* 4012.
8. Castle E, Leidner U, Nowak T, Wengler G, Wengler G, 1986. Primary structure of the West Nile flavivirus genome region coding for all nonstructural proteins. *Virology 149:* 10–26.
9. Hammam HM, Clarke DH, Price WH, 1965. Antigenic variation of West Nile virus in relation to geography. *Am J Epidemiol 82:* 40–55.
10. Price WH, O'Leary W, 1967. Geographic variation in the antigenic character of West Nile virus. *Am J Epidemiol 85:* 84–86.